THE EFFECT OF PURE PROTEIN SOLUTIONS AND OF BLOOD SERUM ON THE DIFFUSIBILITY OF CALCIUM.

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(Received for publication, April 26, 1926.)

I.

INTRODUCTION.

In 1911 Rona and Takahashi (1) studied the diffusibility of serum calcium by "compensatory dialysis." Their method consisted in dialyzing sera against physiological salt solutions containing varying amounts of $CaCl_2$ and observing the calcium content of the sera at equilibrium. They found that the final Ca content of the sera varied, but that in those cases where it did not change, the concentration of Ca in the sera was greater than that in the outside solutions. The difference between these two concentrations was termed the "non-diffusible calcium content of the blood," because it was considered inconceivable that ionized Ca should exist in unequal concentrations on the two sides of the membrane.

These observations have stimulated a large amount of investigation and much speculation concerning the nature of calcium in the blood. Cushny (2) concludes as a result of pressure filtration experiments with serum that no electrolytes except Ca and possibly Mg are combined with proteins and that one-third of the Ca is so held. Neuhausen and Pincus (3), applying the same general method conclude that 30 to 50 per cent of the Ca is non-diffusible. Physicochemical interpretation of such experiments seems difficult with the present state of our knowledge. In 1921, von Meysenbug, Pappenheimer,

Zucker, and Murray (4) confirmed the findings of Rona and Takahashi and showed that in cases of rickets and experimental tetany there is no change in the ratio of Ca in the serum to Ca in the dialysate. This is not in accord with the findings of Cruickshank (5). von Meysenbug *et al.* also showed that changes in CO_2 tension from 17 to 62 mm. with corresponding changes in pH of the dialysate between 7.0 and 7.6 had no apparent influence on the ratios. Neuhausen and Marshall (6) working with a calcium electrode have come to the conclusion that about 80 ± 5 per cent of the Ca is in an unionized form. The correctness of this estimation is open to criticism in view of the fact that no very satisfactory calcium electrode has yet been developed. In 1923, Salvesen and Linder (7), in comparing the Ca content of blood serum and edema fluid found that about 55 to 70 per cent was diffusible (as judged by difference in the concentrations between the two) and that the Ca content of the serum varies directly with its protein per cent. They conclude that decreases in Ca in the blood in nephritis are due to a loss in the non-diffusible Ca, whereas in tetany the decrease is due to a diminution in the diffusible portion as there is no fall in the protein concentration.

Recently, Cameron and Moorhouse (8) have carried on extensive studies on the nature of calcium in blood and spinal fluid. They conclude that 53 per cent of the calcium of the plasma is diffusible, as a result of simultaneous Ca determinations in plasma and spinal fluid. They believe that in tetany the diffusible Ca of the blood increases from 70 to 100 per cent of the serum Ca. This idea is diametrically opposed to that of Salvesen and Linder (7). They believe also that "slowly dissociating" Ca in the blood is *not* in combination with proteins, but with a specific substance termed by them CaX which, under certain conditions, is transformed into another organic (non-protein) compound Y, of large molecular size. Moritz (9), studying the effect of parathyroidectomy on the ratio of "colloidal-diffusible" calcium in rabbits, concludes that there is a somewhat greater loss of diffusible calcium than of colloidal Ca in tetany.

2 years ago the writer (10) showed that the Ca in serum is completely diffusible when the serum is dialyzed against *large* amounts (infinite volume) of physiological salt solution at pH 7.4 but that about 25 to 45 per cent of the Ca is non-diffusible in the absence of

NaCl. It was shown, furthermore, that when serum is dialyzed against large amounts of HCl at pH 3, without the addition of NaCl, the Ca is completely diffusible. The conclusion was drawn that the lack of complete diffusion in the absence of NaCl at pH 7.4 was due to the precipitation of Ca-globulinate. Cameron and Moorhouse state that they were unable to confirm these results but the amount of outside fluid used by them was insufficient to permit complete diffusion. The writer's work did not disprove the presence of undissociated Ca compounds in the blood.

From the foregoing paragraphs, which constitute only a fragmentary review of the literature, it is obvious that much speculation and many contradictions have existed regarding the diffusibility and ionization of serum calcium, and that no comparative studies of pure protein solutions have been made.

The present work was undertaken in the hope of determining whether or not the conditions governing the diffusion of calcium are specific for blood serum or whether more general laws may not apply. For this purpose, the diffusibility of Ca in blood serum has been compared with that in solutions of crystalline egg albumin and serum globulin. The general method employed has been that of dialysis in a closed system similar to that used by Rona and others.

II.

EXPERIMENTAL.

1. *Methods.*—The protein solutions or blood sera were placed in collodion sacs (of 15 to 17 cc. capacity) attached to rubber stoppers. Dilution effects were partially avoided by closing the openings in the stoppers with glass rods. The collodion sacs, with their contents, were immersed in bottles containing 300 cc. of 0.8 per cent NaCl to which were added varying amounts of a 2 per cent solution of $CaCl_2$ (1.5 to 9.7 mM. per litre). *The $CaCl_2$ was always added to the outside solution.* The dialysis time in all of the experiments was 20 to 30 hours. The temperature was kept at 25°C.

The sacs were made of Merck's U.S.P. collodion. After emptying the sacs, they were rotated for 2 minutes and air-dried for 4 minutes, following which they were kept in water. The sacs were always carefully tested for leaks before being used.

Protein determinations were made by the macro Kjeldahl method. The protein figures vary within ±0.15 per cent owing to unequal stretching of the sacs.

Some of the calcium determinations were made on the protein solutions by the Kramer and Tisdall (11) method with the modifications suggested by Clark and Collip (12). Other determinations were made by ashing. There was excellent agreement between these two methods. In the case of aqueous solutions, precipitation of Ca oxalate was brought about by the addition of N/1 $H_2C_2O_4$ and subsequent buffering with a saturated solution of sodium acetate as in the Tisdall and Kramer (13) method for Ca determination in stool and urine. The precipitation was always allowed to go on for at least 4 hours. Determinations were made in duplicate. Blank determinations showed the protein solutions to be Ca-free.

Hydrogen ion concentration determinations were made colorimetrically on the outside solutions and those shown in the tables were made at the completion of dialysis.

2. *Crystalline Egg Albumin.*—This was prepared according to the method of Hopkins (14) and after two washings it was recrystallized once. The albumin was then dialyzed for 48 hours against running tap water and for 6 days more against 10 litres of 0.8 per cent NaCl brought to a pH of 7.4 with $NaHCO_3$. This solution was changed daily. Thymol was used as a preservative.

3. *Serum Globulin.*—This was prepared from ascitic fluid removed at operation from a patient suffering from carcinoma with abdominal metastases. There was no blood in the straw-colored fluid. An equal volume of a saturated solution of ammonium sulfate was added slowly to the fluid and the mixture was allowed to stand for 18 hours. The solution was filtered and the precipitate dialyzed against running tap water for 48 hours; the sediment in the sacs was then removed after the addition of a small amount of ammonium sulfate to complete solubility, and the globulin was reprecipitated. The precipitate was again dialyzed against tap water and then for 6 days against 0.8 per cent NaCl at a pH of 7.4 as in the case of albumin. Before setting up the experiments on the acid side of the isoelectric point, the globulin was first dialyzed against large volumes of physiological salt solution made up in N/1000 HCl. This resulted in some dena-

turization of the globulin. The precipitate was removed before the experiment was begun. This procedure was also carried out in the experiments with albumin.

4. *Blood Serum.*—This was obtained from patients suffering from cardiac insufficiency, lobar pneumonia, or emphysema. In some cases the serum was first dialyzed Ca-free against NaCl solution. All such cases are so indicated in the tables. It appeared to make no difference in the results whether or not previous dialysis had taken place.

III.

RESULTS.

Table I shows the result of dialyzing solutions of egg albumin of various concentrations against solutions of NaCl containing varying amounts of $CaCl_2$. The pH of the outside solution was brought to 7.4 with $NaHCO_3$ before dialysis. It is obvious that the ratio: $\frac{\text{Ca inside}}{\text{Ca outside}}$ is greater than unity and that the ratio increases with the concentration of the protein solution. Table II shows that exactly the same results are obtained when serum globulin is used instead of egg albumin in the collodion sacs. Table III shows that for blood serum, the ratio: $\frac{\text{Ca inside}}{\text{Ca outside}}$ is also greater than 1. This confirms the findings of other investigators (1, 4). The table shows, furthermore, that the ratio varies directly with the protein per cent as in the case of egg albumin or serum globulin.

When egg albumin, serum globulin, or blood serum is dialyzed against 0.8 per cent NaCl in HCl so that the final outside pH lies on the acid side of the isoelectric point of the proteins, it is apparent that the *inverted* ratio: $\frac{\text{Ca outside}}{\text{Ca inside}}$ now becomes greater than unity as may be seen in Tables IV, V, and VI.

Fig. 1 shows the effect of various H ion concentrations on the Ca ratios of egg albumin and two different sera originally at a pH of 7.4. These two sera were not previously dialyzed Ca-free. Serum 1 contained a considerable amount of hemoglobin. Serum 2 was semi-jellied in the most acid sac. The albumin solutions showed increas-

TABLE I.

The Effect of Varying Concentrations of Crystalline Egg Albumin on the Distribution of Calcium at pH 7.35 ± 0.15.

No.	Ca inside.	Ca outside.	Ratio: $\frac{\text{Ca inside}}{\text{Ca outside}}$.	
	mM.	mM.		
1	2.23	1.63	1.37	Protein per cent = 7.1.
2	4.33	3.0	1.44	
3	5.73	3.85	1.49	
1	2.03	1.65	1.23	Protein per cent = 3.5.
2	3.9	2.9	1.34	
3	5.23	4.05	1.29	
1	1.75	1.58	1.11	Protein per cent = 1.5.
2	3.4	3.13	1.09	
3	4.48	4.0	1.12	

TABLE II.

The Effect of Varying Concentrations of Serum Globulin on the Distribution of Calcium at pH 7.4 ± 0.1.

No.	Ca inside.	Ca outside.	Ratio: $\frac{\text{Ca inside}}{\text{Ca outside}}$.	
	mM.	mM.		
1	2.2	1.4	1.57	Protein per cent = 7.9.
2	4.1	2.95	1.39	
3	5.3	3.8	1.39	
1	2.12	1.58	1.34	Protein per cent = 5.7.
2	3.05	2.37	1.29	
3	4.0	3.08	1.30	
4	5.2	4.1	1.27	
1	1.95	1.52	1.28	Protein per cent = 2.7.
2	3.55	3.18	1.12	
3	4.7	4.08	1.15	
1	1.45	1.23	1.18	Protein per cent = 1.2.
2	3.28	3.08	1.06	
3	4.43	4.13	1.07	

TABLE III.

The Effect of Blood Serum on the Distribution of Calcium at pH 7.35 ± 0.15.

No.	Ca inside.	Ca outside.	Ratio: $\frac{\text{Ca inside}}{\text{Ca outside}}$.	Protein per cent.
	mM.	mM.		
1	3.53	2.25	1.57	7.8
2	3.68	2.3	1.60	7.5
3	4.88	3.18	1.53	7.0
4	3.38	2.18	1.55	6.9
5	2.23	1.63	1.37	6.3
6 x	5.2	3.45	1.51	6.1
7 x	9.73	6.98	1.39	6.1
8 x	2.35	1.53	1.54	6.1
9 x	3.38	2.13	1.59	6.1
10 x	4.25	2.85	1.49	6.1
11 x	5.6	3.55	1.58	6.1
12 x	2.98	1.78	1.67	5.9
13 x	2.28	1.70	1.34	5.3
14 x	5.53	4.25	1.30	5.3
15 x	2.3	1.65	1.39	5.2
16 x	5.3	4.65	1.45	5.2
17	2.23	1.78	1.25	4.6
18	4.05	3.25	1.23	4.0
19	3.80	3.25	1.17	2.7
20	2.18	1.93	1.13	2.3
21	3.45	3.28	1.05	1.5

x signifies that the serum was dialyzed Ca-free against 0.8 per cent NaCl at pH 7.4 before being used in the experiment in which $CaCl_2$ was added to the outside solution as usual. Serum dilutions were made with 0.8 per cent NaCl at pH 7.4.

TABLE IV.

The Effect of Crystalline Egg Albumin on the Distribution of Calcium at pH 3.8 to 4.0.

No.	Ca outside.	Ca inside.	Ratio: $\frac{\text{Ca outside}}{\text{Ca inside}}$	
	mM.	mM.		
1	1.63	1.45	1.12	
2	3.08	2.95	1.04	Protein per cent = 5.3.
3	4.23	3.9	1.08	
1	1.75	1.55	1.13	
2	3.3	3.13	1.06	Protein per cent = 5.2.
3	4.45	3.98	1.12	
1	1.7	1.53	1.11	
2	3.25	3.03	1.07	Protein per cent = 2.8.
3	4.18	3.73	1.12	

TABLE V.

The Effect of Serum Globulin on the Distribution of Calcium at pH 3.6 to 4.0.

No.	Ca outside.	Ca inside.	Ratio: $\frac{\text{Ca outside}}{\text{Ca inside}}$.	
	mM.	mM.		
1	2.7	2.53	1.07	Protein per cent = 4.1.
2	3.78	3.68	1.03	
1	1.53	1.48	1.03	
2	3.03	3.0	1.01	Protein per cent = 5.9.
3	4.1	3.8	1.08	

pH measurements here were estimated from similar experiments with egg albumin.

TABLE VI.

The Effect of Blood Serum on the Distribution of Calcium at pH 3.6.

No.	Ca outside.	Ca inside.	Ratio: $\frac{\text{Ca outside}}{\text{Ca inside}}$.	Protein per cent.
	mM.	mM.		
1	1.73	1.53	1.13	5.2
2	4.35	3.73	1.17	5.5
1	1.65	1.45	1.14	5.9
2	4.2	3.83	1.10	6.4

The sera used in these experiments were first dialyzed Ca-free against large volumes of 0.8 per cent NaCl in N/1000 HCl.

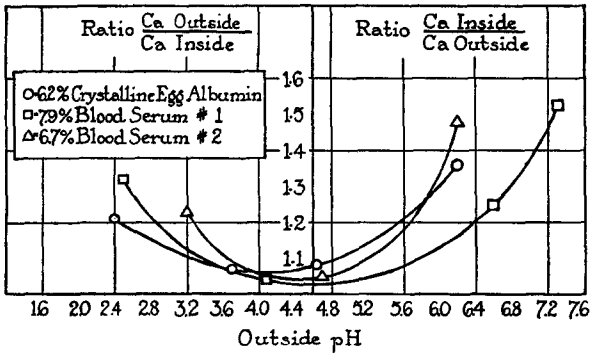

FIG. 1. The influence of various H ion concentrations on the distribution of calcium.

ing turbidity with increasing acid concentration. The original outside Ca concentration in this series of experiments was 4.0 mM. per litre. The curves show clearly that the ratio: $\frac{\text{Ca inside}}{\text{Ca outside}}$ decreases with increasing H ion concentration over the range studied and that the ratio: $\frac{\text{Ca outside}}{\text{Ca inside}}$ becomes greater than 1 on the acid side of the isoelectric point of the proteins.

IV.

DISCUSSION.

From the experimental data presented it becomes apparent that the forces which determine the diffusibility of calcium in blood serum are the same qualitatively and in general quantitatively as those which determine its diffusibility in pure solutions of egg albumin and serum globulin. It appears that the explanation for the behavior of calcium in serum and in the other protein solutions studied is to be found in the newer concepts of colloidal behavior as they have been outlined in the work of Jacques Loeb (15) and more recently extended by Northrop and Kunitz (16). The idea that ion concentrations must be unequally distributed on two sides of a membrane when that membrane is permeable for all but one of the ions in the system is no longer novel but was, of course, not appreciated when Rona and Takahashi (1) first undertook their studies on the diffusibility of calcium. While no membrane potentials were determined and no measurements of Ca or Cl E.M.F. were made in the experiments reported in this paper, the findings appear to be quite in harmony with the Donnan theory, assuming, as do Northrop and Kunitz (16) for Zn and other ions, the formation of a complex Ca-protein ion. Northrop and Kunitz have shown that there is definite evidence in favor of the idea of complex ion formation between proteins and other ions rather than changes in activity coefficients of the ions by the proteins. This is the explanation offered by them for discrepancies between total analytical concentrations and values determined by E.M.F. measurements.

It becomes quite obvious from the rather close quantitative parallelism between the behavior of pure protein solutions and blood serum

that if there are any other factors in the latter influencing the diffusibility of Ca, their rôle must indeed be very small. The assumption made by Cameron and Moorhouse (8) that the "non-diffusible" calcium of blood plasma results from the combination of Ca and some substance, other than protein, thus appears to be unnecessary.

The observation made by Salvesen and Linder (7) that the serum Ca in edematous nephritics falls as the protein concentration diminishes is in harmony with the experiments presented in this study and finds its explanation in the fact that the ratio: $\frac{\text{Ca inside}}{\text{Ca outside}}$ varies with the protein concentration as is demanded by the Donnan theory.

V.

SUMMARY.

1. A comparative study has been made of the diffusibility of calcium in solutions of crystalline egg albumin, serum globulin, and human blood serum.

2. In all three of these solutions, at pH 7.4, molal Ca concentrations within the membrane are greater than the calcium concentrations in the outside solutions, quite in accordance with the Donnan theory.

3. At pH 7.4, the ratio of $\frac{\text{Ca inside}}{\text{Ca outside}}$ varies directly with the protein concentration whether the solution be one of egg albumin, serum globulin, or blood serum. This is also in accordance with the Donnan theory.

4. On the acid side of the isoelectric point of the proteins, the concentration of Ca outside becomes greater than the concentration in the solution of blood serum or pure protein, as is demanded by the Donnan theory.

5. The magnitude of the Ca ratios on the alkaline and acid sides of the isoelectric points is probably the resultant of the Donnan equilibrium and the formation of complex Ca-protein ions. Northrop and Kunitz have shown the probability of the existence of such ions in the case of Zn^{++}, K^{+}, and Li^{+}, where satisfactory electrodes have been developed for E.M.F. measurements.

VI.

CONCLUSIONS.

Comparative studies of the effect of pure proteins and blood serum on the diffusibility of calcium indicate clearly that the behavior of calcium finds its explanation in the Donnan theory of membrane equilibria slightly modified by the formation of complex Ca-protein ions.

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